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The dynamic morphology of the nephron: Morphogenesis of the "protein droplet"

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Since the time of Virchow, a number of observers have noted the appearance of hyaline droplets in the epithelial cells of the proximal convolution of the nephron. These intracellular bodies (Fig. 1, *A* and *B*) were considered by von Möllendorf and Stohr [1] and Volhard and Fahr [2] as clear evidence of renal damage or as sequelae or a concomitance to severe proteinuria, and the process respectively termed "Speicherung" and "athrocytosis." However, an approach to a mechanistic and physiologic description of this interesting and complex organelle was initiated only 30 years ago. The impetus for this accumulation of dynamic information concerning the evolution of droplets in renal cells (a secondary lysosome) emanated primarily from the laboratory of the esteemed morphologist Jean Oliver [3–12].

Although the hyaline droplets found in the cells of the proximal convoluted tubule were usually associated with malfunction of the kidney, e.g., massive proteinuria, Oliver, MacDowell and Lee [7] recognized the functional nature of the process. They said, "The intracellular phenomena that accompany proteinuria previously described under the various disparate categorical entities of absorption, cloudy swelling, athrocytosis, hyaline droplet formation, and 'diseases of the kidney tubule,' or as evidence of general disturbance of 'metaprotein metabolism,' become thus an integrated and unified process." They also went on to say [7], "The changes described in the renal cells are not thus common to the daily, i.e. 'physiological,' life of a rat. However, the alterations we shall describe are reversible. . . ."

The statements were prophetic, but required extension, since the processes are common to the ordinary life of the renal cell but cannot always be visualized with ordinary bright-field microscopy. Oliver and his co-workers realized this possibility and said that appearance of droplets can be considered an abnormal modification of a process that is ordinarily carried on by the cells of the proximal convolutions of the nephrons, namely the reabsorption and disposal of plasma proteins.

Allen [13] presented an argument which forcibly posed the belief that droplets were primarily related to disease, that the glomerulus was an idealized ultrafilter, that the proximal cells did not absorb protein and that the droplets represent a mild (or severe), potentially reversible, osmotic reaction, but may represent the initial phase of a necrotizing process. He compared the formation of droplets to the same degenerative processes encountered in the tubule following administration of potassium dichromate or diethylene glycol. Both of these substances are cellular poisons and their effects are not related to the process of protein absorption. The major efforts concerning the physiological definition for the formation of droplets emanated from work accomplished in the laboratory of Jean Oliver. It was this work that was extended by Novikoff [14, 15] and Miller [16], which served as the basis for a clearer understanding of this particular function of the cell of the proximal convoluted tubule.

The observations of Oliver [3] stimulated Smetana [17], who in 1947 injected a number of different species of animals with the dye 2 naphthol-3-disulfonic acid, bound to various homologous and foreign proteins. This complex passed through the glomerulus and was absorbed; it could be readily seen in renal cells of the proximal convolution. The protein-dye complex was not detected in the urine. When the kidney was prepoisoned with uranyl nitrate, there were no evidences of absorption of the complex by the cells. Smetana

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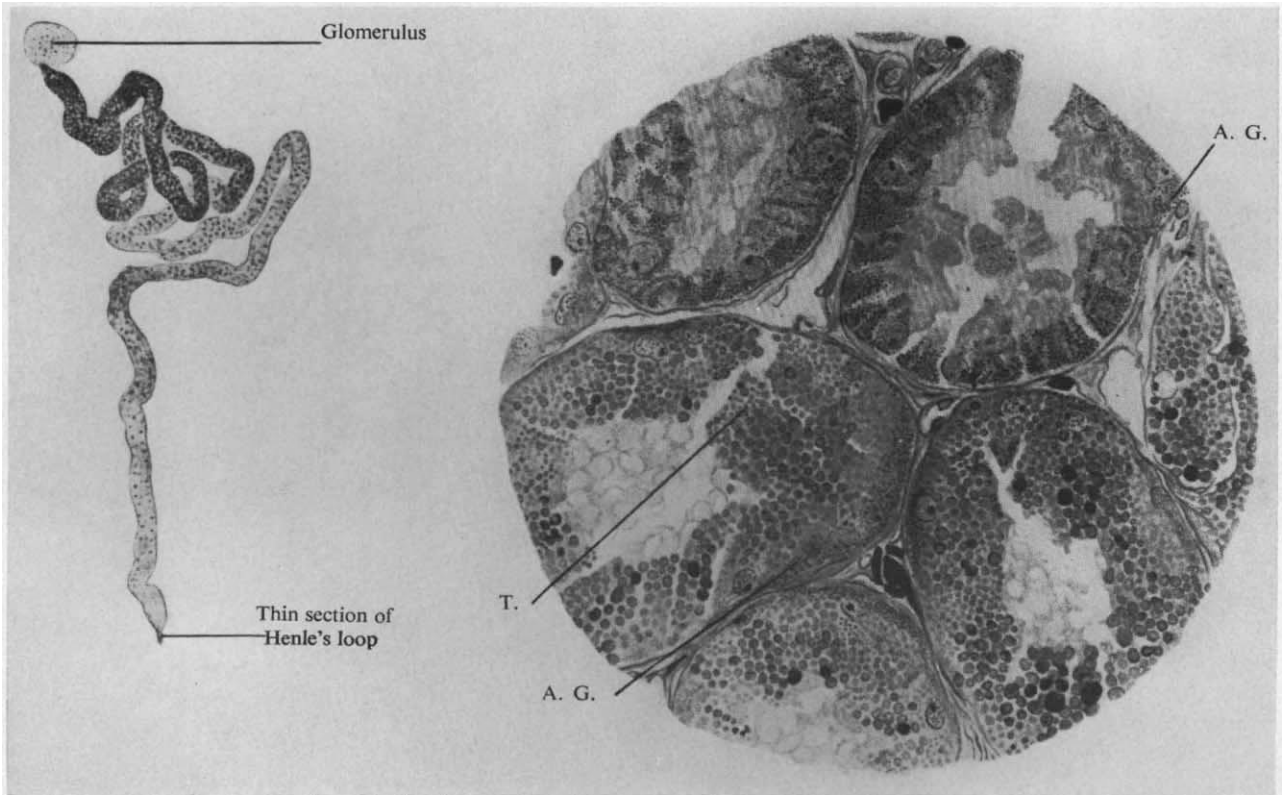


Fig. 1. *A, Drawing from 1915 manuscript by V. Möllendorff. B, Drawing of a histological section from the kidney of a patient with nephrosis.* "These cells are full of droplets." (T.) droplets; (A.G.) Altmann granules. (From Volhard and Fahr [2], Table II, Plate 4, oil immersion.)

concluded from these experiments that the glomerulus permitted those protein complexes to be filtered, that once in the luminal fluid they were absorbed by the cells and that these phenomena were evidence of normal function.

Oliver [3] believed that the glomerulus normally permitted some leakage of protein and, under the conditions of an elevated intravenous concentration of protein, a greater number of the molecules would be present in the glomerular filtrate. Consequently, he and his associates injected a [4, 6–8] mixture of egg white and saline solution intraperitoneally into rats, and showed by histological section and the exquisite technique of microdissection of the nephron that there were gram-positive droplets primarily located in the cells of the proximal convolution. These droplets were first detected at the apical end of the cell; they moved to the basilar side, where they finally disappeared. The peak extent of droplet formation observed in the cells occurred approximately 12 to 18 hours after injection of the egg white. A similar phenomenon of droplet formation was disclosed when large amounts of different amino acids were injected intravenously.

Oliver's conception of the process was that the protein (or amino acid) filtered through the glomerulus, and that it was absorbed from the luminal fluid by the renal cells. The formation of the droplet was conceived of as evidence of overtaxing the existing cellular machinery. With time, digestion or utilization of the molecule took place, with eventual disappearance of the droplet. Others in Oliver's laboratory [10–12] showed that these droplets contained elevated concentrations of egg white or amino acids. Consequently, the concept was [7] that the droplet was, "... a structural aspect of a mechanism which brings together enzyme and substrate for a resulting metabolic modification of the absorbed material."

Oliver saw the need for a source of enzymes in the droplet; his observation, from histological sections and dissections, indicated that the source of metabolic machinery was derived from the presumed dissolution of mitochondria. This conclusion was based on the fact that he could not see mitochondria during peak formation of droplets, and that he knew there must be a source of enzyme for the droplet. In addition, evidence from electron microscopy [18] was purported to substantiate this supposition. Critical histochemical work in his laboratory was accomplished by Straus

[12, 19, 20]. Straus purified the droplets and found six times more egg white in association with particles than in any other intracellular organelle. He utilized injected horseradish peroxidase, an easily identifiable foreign protein, so that he could readily follow formation of droplets. With this unique technique, he was able to confirm Oliver's observations and conclusions concerning formation of droplets in renal cells.

In 1955, deDuve et al [21] reported their concept of the remarkable lysosome, an intracellular digestive particle containing a number of acid hydrolases. The concept of the lysosome was developed from observations on rat liver, and indicated a biologically meaningful association of enzymes with the particle. The latency of the activity of these enzymes can be attributed to the effective membrane-barrier of the particle separating it from the intracellular environment. It was these observations that were the major stimulus to the clarification of the process of droplet formation in the kidney.

Among the many acid hydrolases contained in the lysosomes was acid phosphatase, an enzyme which was easily identifiable by histochemical techniques [14, 15, 22]. Straus [19, 20, 23, 28] utilized a combination of measurements of horseradish peroxidase and acid phosphatase to follow the evolution of the droplet. He showed [19, 20] that droplets in the apical portion of the cell were negative for acid phosphatase, but contained horseradish peroxidase; these particles he termed phagosomes. They appeared 30 minutes after injection at the base of the brush border, and were separate from the lysosomes which contained acid phosphatase. Within one to three days following injection, there was a coalition between these particles and the new particle termed a phago-lysosome. After one to three additional days, there was a loss of evidence of horseradish peroxidase and a persistence of the acid phosphatase. The critical historical aspect of this process was that the mitochondria did not disappear, but could have been observed during the process. They offered the possibility of furnishing energy for the entire phenomenon. Novikoff [14, 15] stated that under extreme conditions (i.e., hydronephrosis) giant droplets, which he termed cytolysosomes, could form and also obscure the view of all intracellular particles. He also showed that droplets have a single membrane and are formed from pinocytotic vacuoles, containing the protein, which originate via canalicular structures between adjacent microvilli. These small vacuoles tend to fuse. Miller [16] accomplished the critical survey of the problem which put all argument to rest. He reiterated that droplets appear in the presence of a great excess of protein and indicate, as Oliver said, an accumulation of material that cannot be readily metabolized by the cell. In order to answer the question

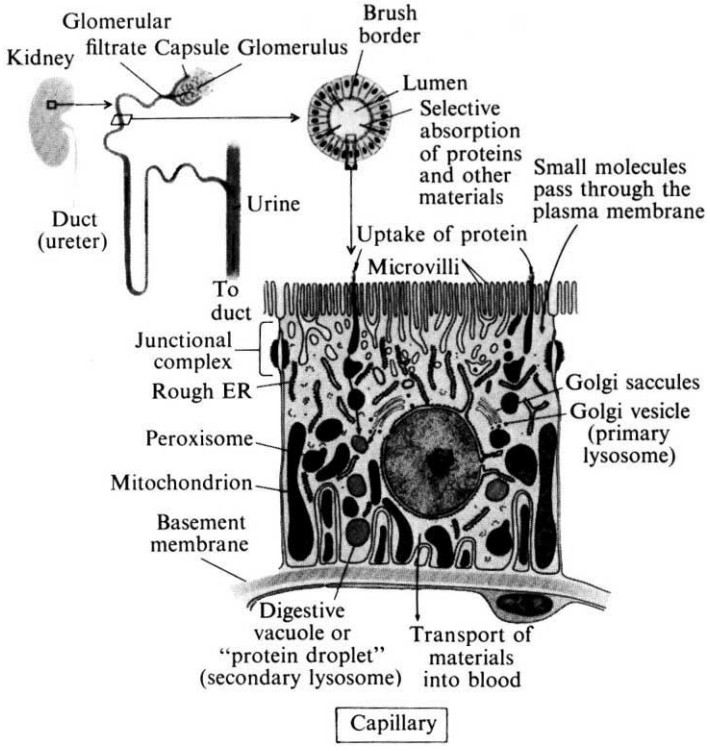


Fig. 2. *Drawing of an epithelial cell from the proximal convoluted tubule of the kidney, indicating the processes involved in the fate of absorbed protein.* (From *Cells and Organelles* by Alex B. Novikoff and Eric Holtzman. Copyright © 1970 by Holt, Rinehart and Winston, Inc. Reproduced by permission of Holt, Rinehart and Winston, Inc.) [25].

of the role of mitochondria and the ontogenesis of the droplet, he injected purified hemoglobin intraperitoneally and then followed the process for four days with the electron microscope.

Miller stated [16] that hemoglobin entered a duct formed by tubular invaginations and was transported in vacuoles which fill and are surrounded by a single-layered membrane which is then pinched off. He indicated that, as Oliver showed, this must be a continuous process which probably not only demands the energetic participation of mitochondria, but also that there is a role for the Golgi in membrane formation. These droplets stay separated; however, they are often found in close approximation to the mitochondria. This phenomenon gives the potential for an erroneous conclusion which could not be determined without the advantage of the powerful procedures of modern electron microscopy. It was rare and probably accidental to encounter a mitochondrion trapped within a droplet.

It is now possible, approximately 30 years later, to synthesize clearly the entire process of protein or amino acid droplet formation and disposal (Fig. 2). When a large amount of protein or amino acid has been filtered through the glomerulus and is presented to the cells of the proximal convolution, it is taken up by the process of "endocytosis" (a name suggested by deDuve [26]) into vacuoles. These vacuoles, containing the protein (phagosomes) coalesce with the primary lysosomes or with secondary lysosomes (phago-lysosomes) to form a complex secondary lysosome. The protein will then be hydrolyzed in these particles and the resulting hydrolysate can be utilized by the cell or enter the bloodstream. The primary lysosome with its contents is undoubtedly formed by mutual participation between the synthesis of enzymes on the ribosomes and contribution of membranous materials from the Golgi.

The process is a normal, exquisite phenomenon encountered in most cells and serves as a basis for intracellular digestion and handling of an overload of a substance or the presence of a foreign material.

Oliver [4], who made the basic observations in the renal cell and arrived at many accurate conclusions, was correct when he stated, "Apparently the presence of absorption droplets in the renal cells is therefore not evidence of damage but proof of a certain functional and structural intactness." Presumably when this mechanism fails, there is a potentially irreversible damage.

Ultrastructural observations

Background; Experimental data. Much of the controversy over hyaline droplets was dissipated by their morphologic identification and by studies of their morphogenesis in experimental animals. Ultrastructural studies of tubular resorption demonstrated that the droplets, which took up injected dyes and enzymic markers and which had been shown by Straus [27, 28] also to contain acid hydrolases, were single membrane-bound vesicles identified as lysosomes by Novikoff [14, 15]. It became apparent that droplets did contain protein resorbed through a process of endocytosis. It also became quite apparent that mitochondria did not participate directly in the process and that they retained their normal appearance. The experimental studies have provided the guide to an understanding of proteinuria and droplet-formation in clinical renal disease.

In experimental studies of tubular resorption, using hemoglobin [16, 31–33], colloidal substances [34], radiolabeled albumin [35], horseradish peroxidase [36, 37], cytochrome c [38] and tyrosinase [39], the tracer could be identified within the apical cytoplasm of proximal tubular cells. Substances injected intravenously [31–34, 36–39] are seen within minutes in tubular lumens and among microvilli of the brush border. The marker may be present in increased concentration on brush border membranes, suggesting that initial surface adsorption plays a role in the process of tubular absorption [36]. The injected materials are taken into the cells through apical invaginations at the bases of the microvilli and are passed through a network of small tubules and vesicles to be concentrated in the somewhat larger apical vacuoles (Fig. 2). The precise manner in which material is transported from the extracellular invaginations to the intracellular vacuoles has not been completely resolved. The invaginations and the subjacent tubules and small vesicles are membranous structures lined both inside and outside by a fuzzy coat similar to that seen on the extracellular surface of brush border. The apical vacuoles are also lined internally, and intracellular tubules, often more than one in the plane of section, commonly are joined directly to the larger vacuoles [16, 33, 36]. These structures appear to be part of the same transport system, with functional continuity, but anatomic continuity of the entire system has not in fact been established. It has been suggested, therefore, that pinocytotic vesicles, arising from superficial apical invaginations and tubules, are pinched off and migrate to a somewhat deeper cytoplasmic zone above the nucleus where they fuse to form vacuoles [16, 29, 35]. Conceivably, the vacuoles themselves participate as the transport vesicles [32], losing their external fuzzy coats and presumably fusing to result in considerable enlargement. Perhaps apical tubules function

intermittently, appearing and opening as necessary to transport microboluses of material from surface invaginations to underlying vacuoles. By whichever mechanism, then, the material, apparently unchanged, is passed through the tubules or vesicles to a smaller number of larger vacuolar structures, where the material accumulates.

The absorbed material seems to be concentrated in the apical vacuoles, which are seen to contain scattered flocculent densities within an hour of intravenous injection. The vacuoles increase in number and enlarge in size to several microns, and by light microscopy are visible just beneath the brush border. They apparently migrate from there, streaming toward the region of the nucleus [32], losing en route the membrane-associated internal filamentous coating and acquiring acid hydrolase activity. The morphologic differentiation of vacuoles, "absorption droplets," and secondary lysosomes is imprecise, and the existence of transitional forms has been assumed [33]. Cytoplasmic bodies, identifiable as lysosomes by demonstration of acid hydrolase activity, increase in number and density within a few hours after injection and are associated with the injected label [35, 40]. The transfer of acid hydrolases to vacuoles or droplets [20] might be accomplished by fusion with primary lysosomes, with preexisting secondary lysosomes or acid phosphatase-positive cytoplasmic bodies, or perhaps with enzyme-containing Golgi vesicles. The fusion of droplets and lysosomes seems to have been visualized by Ericsson [32]. The terms "protein droplet" and "absorption droplet" have been used to indicate the vacuolar structure containing resorbed protein, a usage that does not necessarily take into account the acquisition of acid hydrolases and transformation to a secondary lysosome.

Absorbed protein undergoes degradation or digestion within the secondary lysosome, in the instance of hemoglobin reabsorption, for instance, leaving traces of ferritin and, eventually, hemosiderin [16, 30, 32]. The digestive process ordinarily proceeds rapidly and is well-advanced within 12 to 24 hours [16, 32], an observation of importance in evaluating the accumulation of large numbers of very large resorption droplets in patients with chronic proteinuria. The digestion of previously injected, labelled protein (ribonuclease) is seen in minutes *in vitro* in lysosomal preparations [41].

Clinico-pathologic observations. The observations in experimental animals can be applied to the problems of protein resorption and droplet formation in the renal tubules from the human. Electron microscopic study of renal biopsy specimens in proteinuric states [41], as compared with normal proximal convoluted tubules [42, 43], has shown an increase in vacuolar structures (Fig. 3). Apical tubules and vesicles are numerous, and the coated apical vacuoles are relatively large. Considerable variation is present among neighboring cells, and both the subapical and central portions of the cells contain droplets of varying size and density [41, 44] (Fig. 4, *A*). Mitochondria have a normal appearance, and there are numerous profiles of smooth endoplasmic reticulum and Golgi complexes. The picture may be obscured by considerable tubular distortion and cytoplasmic disruption that follow the biopsy procedure and fixation by immersion.

Differences in the size and content of apical tubules and vesicles suggests either heterogeneity [33] or intermittence of function. Tubules are frequently joined to vacuoles in a manner indicating direct communication (Fig. 4, *B*). The tubules contain material of variable density, but the superficial, internally coated apical vacuoles appear to be distended by electron-lucent or granular material. Some small apical vesicles may be tubular profiles in cross-section. Others, however, seem to be unrelated to tubules and can be seen clustered at or streaming from the apical invaginations at the depths of the brush border (Fig. 4, *C*). The material(s) within these structures may not be readily identifiable in such complex disease states as the nephrotic syndrome, but it does appear that resorbed substances can be transported into the cell in more than one fashion.

Vacuoles in the subapical zone and deeper cytoplasm contain granular and flocculent densities that may entirely fill the vacuole. The vacuoles seem to lose their internal fuzzy coats, and precise differentiation of apical vacuoles and "protein droplets" is not achieved (Fig. 5). Vacuoles in the midportions of cells contain membranous figures and amorphous densities, in addition to granular and flocculent material. These vacuoles can be regarded as secondary lysosomes. They form by fusion of absorption vacuoles and either primary or preexisting secondary lysosomes (Fig. 6). The membranous and dense material may be residues of previously digested material.

We have observed that biopsy specimens taken early in the course of the idiopathic nephrotic syndrome do not often contain large accumulations of droplets. Scattered droplets are seen by light and electron microscopy, and the number varies from cell to cell. The large numbers of hyaline droplets seen by light microscopy are more often associated with long-standing proteinuria and with end-stage renal disease. They are seen commonly, of course, in autopsy material, and we have also had the opportunity of studying several kidneys removed by nephrectomy from patients

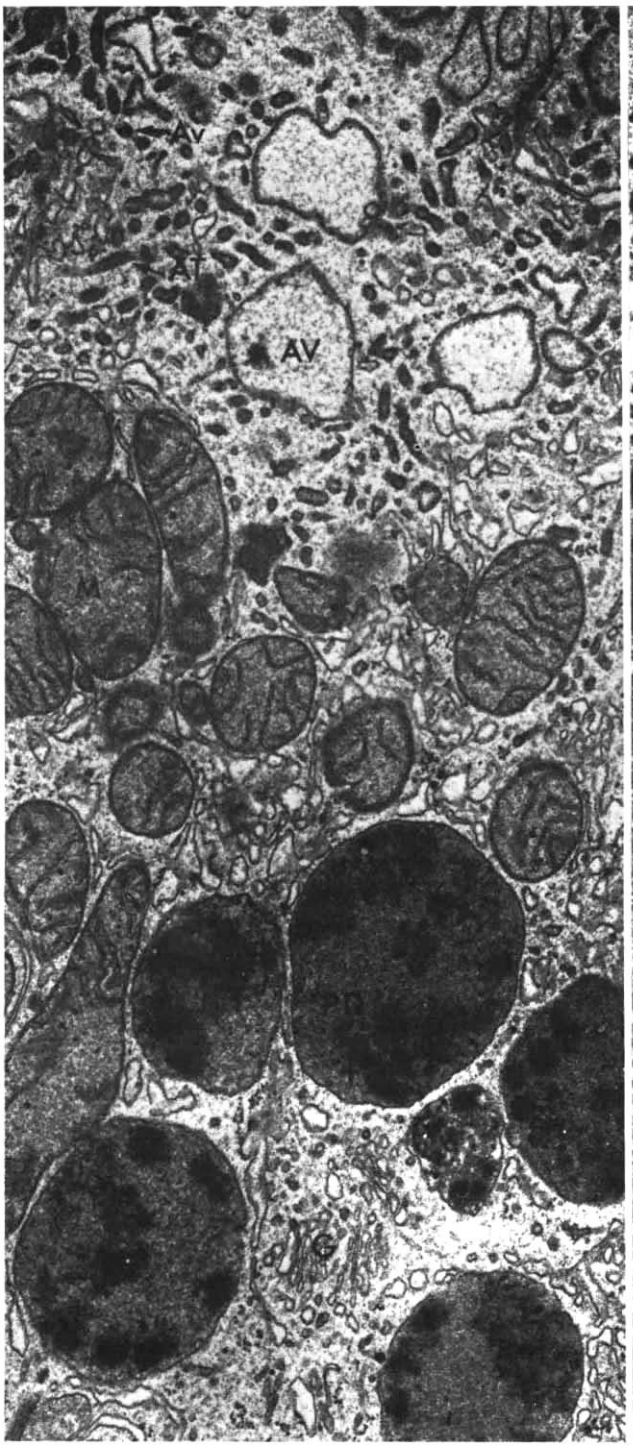


Fig. 3. *Apical and middle portions of proximal tubular epithelial cell, showing network of apical tubules (AT), apical vesicles (Av), and apical vacuoles (AV) (× 19,000).* The midportion of the cell contains numerous protein droplets (PD) of irregular density, some of them clustered around the Golgi apparatus (G). Mitochondria (M) are normal (36-year-old man with proteinuria of one year's duration and the nephrotic syndrome for one month.)

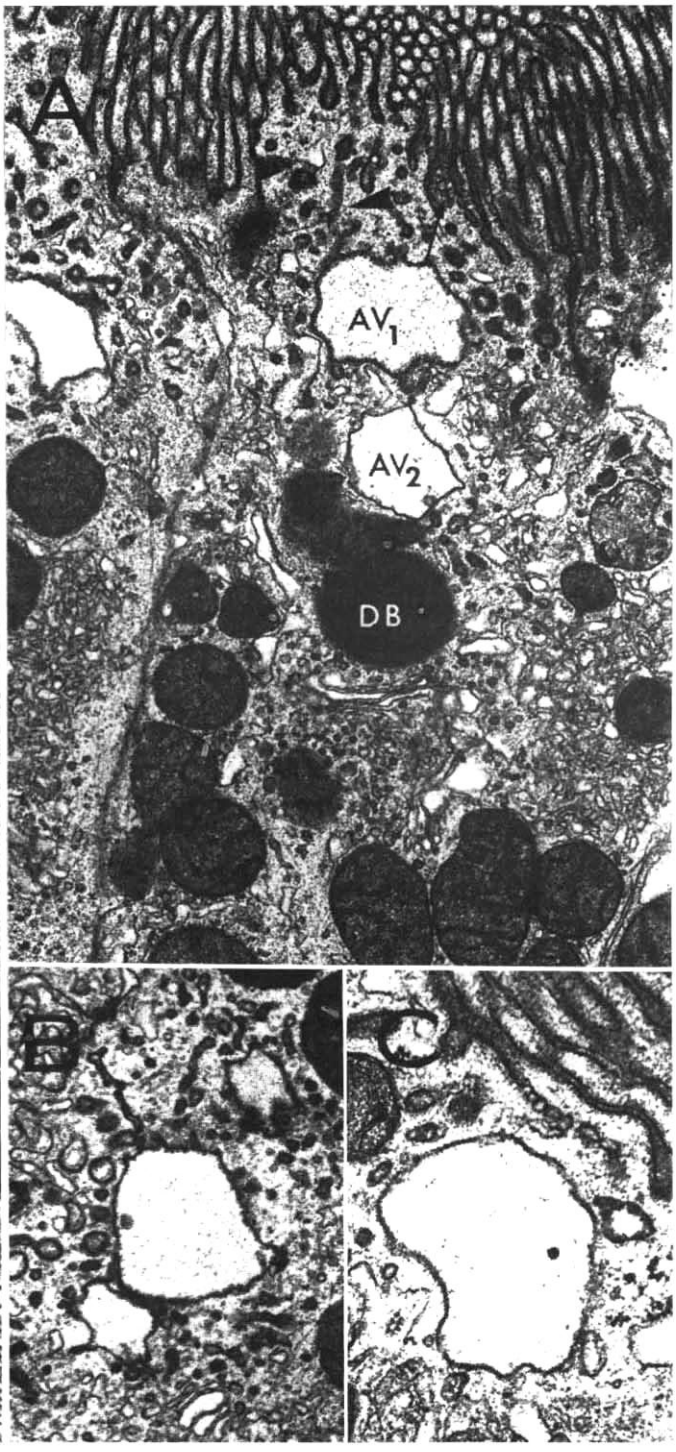


Fig. 4. *Apical region of tubular cell to show relationships of apical tubules, apical vesicles, and larger apical vacuoles. A, An apical vacuole (AV_1) connects with several apical tubules, one of which (►), cut tangentially, seems to have a relatively long and tortuous course (× 19,000.)* Note also clusters of vesicles (→) at base of brush border. Deeper vesicle (AV_2) has partially lost its internal coating and is in close proximity to a dense cytoplasmic body (DB) (30-year-old man with nephrotic syndrome of seven years' duration). *B, Apical vacuoles showing continuity with numerous tubules (15,600) (same patient as in Fig. 3). C, Apical region of cell showing line of small vesicles streaming from invagination at base of brush border (× 32,000) (same patient as Fig. 4, A).*

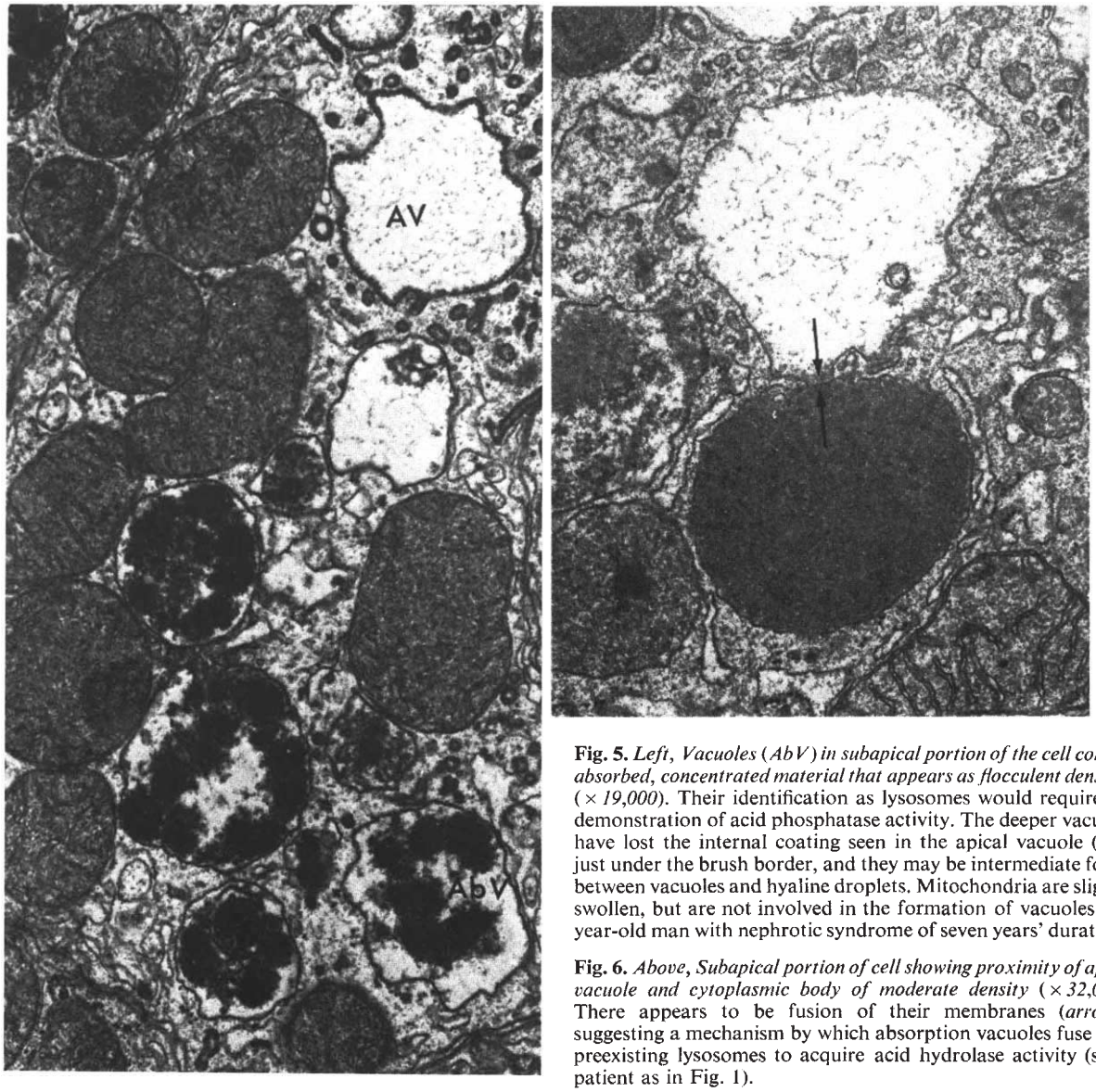
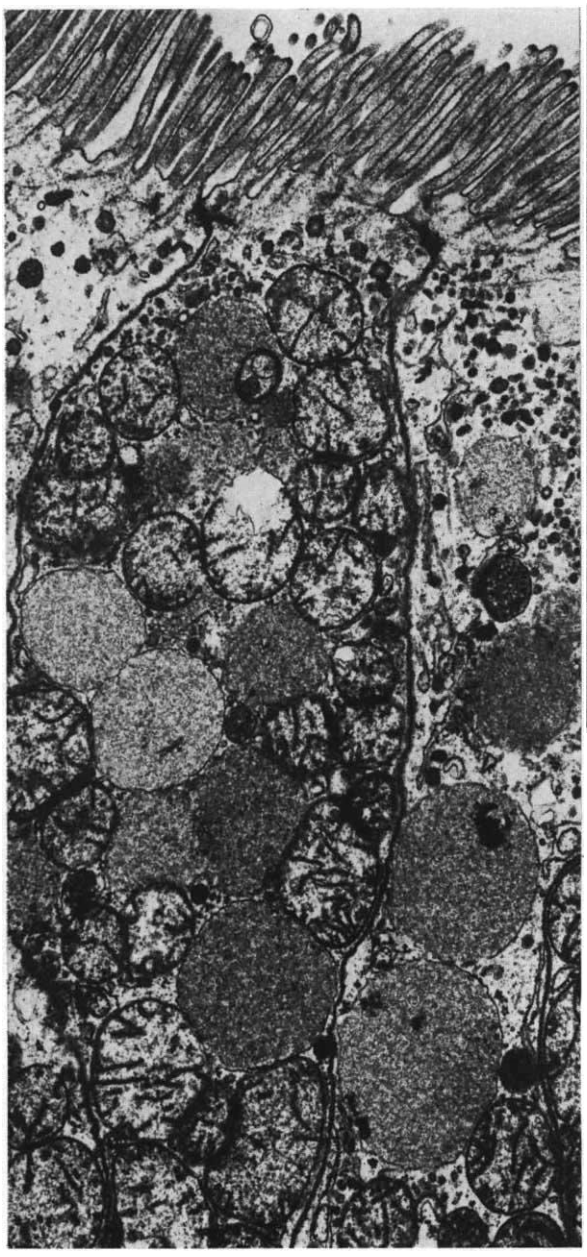


Fig. 5. *Left, Vacuoles (AbV) in subapical portion of the cell contain absorbed, concentrated material that appears as flocculent densities (× 19,000).* Their identification as lysosomes would require the demonstration of acid phosphatase activity. The deeper vacuoles have lost the internal coating seen in the apical vacuole (AV) just under the brush border, and they may be intermediate forms between vacuoles and hyaline droplets. Mitochondria are slightly swollen, but are not involved in the formation of vacuoles (30-year-old man with nephrotic syndrome of seven years' duration).

Fig. 6. *Above, Subapical portion of cell showing proximity of apical vacuole and cytoplasmic body of moderate density (× 32,000).* There appears to be fusion of their membranes (*arrows*), suggesting a mechanism by which absorption vacuoles fuse with preexisting lysosomes to acquire acid hydrolase activity (same patient as in Fig. 1).

with chronic renal failure. The cells lining the proximal convoluted tubules are filled with large vacuoles containing material of medium, granular density (Fig. 7). There does not appear to be a loss of mitochondria, which might show other degenerative changes, but the cytoplasm appears otherwise to be considerably simplified. Small vesicles are present in the apical cytoplasm, but tubules seem to be diminished in number, and apical vacuoles are few. The cells seem, therefore, to be less active in absorbing material and in transporting it into the cytoplasm. The endoplasmic reticulum and Golgi complexes also seem to be diminished, and the droplets have a more static appearance. They are present at all levels of the cell without the polarization that has morphologically suggested movement from apex to deeper cytoplasm. Some droplets contain membranous material or residues, and this seems to be variable. Nonetheless, the relative paucity of dense membranous residue suggests that the rate of protein digestion and turnover may be low at this stage of the disease. Perhaps the tubular capacity of handling protein has been quantitatively exceeded; perhaps tubular function has been impaired by progressive cellular damage over the course of the disease. Could tubular function have been impaired, as suggested by Oliver, by a surfeit of protein, causing tubular "indigestion"?

Renal tubular alterations in multiple myeloma are also associated with resorption of protein from the tubular lumens. Resorption droplets similar to those



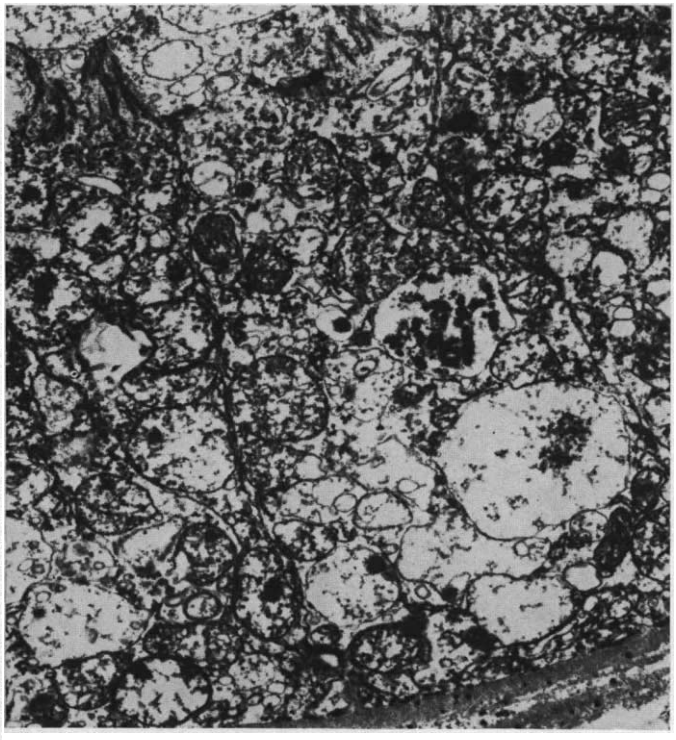


Fig. 7. *Left, Proximal tubular cell in chronic glomerulonephritis, with renal failure showing numerous protein droplets, some of them containing membranous electron-dense residues* (× *16,500*). Apical tubules and vacuoles in the superficial cytoplasm are reduced in number, suggesting that protein resorption is now diminished. Organelles elsewhere in the cell also seem to be reduced in number, perhaps a degenerative change, imparting to the cytoplasm a "simplified" appearance (pretransplantation nephrectomy specimen in a 33-year-old man with chronic glomerulonephritis.)

Fig. 8. *Above, Proximal tubular cells in autopsy specimen of a patient with multiple myeloma dying of acute renal failure* (× *8,100*). Note large vacuoles in midportion of cell containing dense fibrillary material. There appear to be, apart from autolytic changes, tubular damage with partial loss of brush border, simplification of apical cytoplasm and calcification of basement membrane (specimen provided through courtesy of Dr. C. Craig Tisher, Duke University).

seen in nephrotic syndrome are observed in epithelial cytoplasm (Fig. 8), and localization of lambda and kappa chains in tubular droplets has been demonstrated by specific immunofluorescence [46]. In addition, the occasional finding within tubular cells of inclusions and crystals of fibrillary protein [47, 48] appears to be related to the intracellular accumulation of resorbed material. Whether such accumulation results from "indigestibility" of the protein or secondary failure of the lysosomal system is not clear. Conceivably, prolonged proteinuria results in tubular damage and secondary defects of tubular function to account for the occasional occurrence of Fanconi's syndrome of glucosuria, phosphaturia and aminoaciduria in cases of myeloma [47].

Acknowledgments

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